ISOLATION OF AN INHIBITORY SUBSTANCE FROM PLANTS[1]

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INTRODUCTION

The isolation of lytic and inhibitory substances from animal sources by various investigators led the writers to a study of diseased plants in order to determine whether or not such substances were found in association with plant pathogens.

It seems needless to review the literature on bacteriophage since so many recent articles have included extended reviews. A number of investigators have found bacteriophage present in the intestines of man and animals in association with disease organisms. It is generally believed that bacteriophage develops under a diseased condition, adapts itself to the causative organism, and where a recovery results, it develops a high lytic power, causing a lysis of the causative organism.

A survey of the available literature failed to reveal any work of this nature on plant diseases; hence the present work.[2]

Soft rot of cabbage was selected for study, as it was easily obtained. A cabbage showing soft rot was placed in a large culture dish and allowed to decompose until a considerable quantity of liquid material had collected. At this point, loop dilution plates were made in order to isolate the rot-producing organisms. As the plates showed practically a pure culture, several colonies were fished and planted on agar slants. One of these cultures was used for all the work carried

TABLE I.—*The effect of the cabbage-rot filtrate on different organisms* [a]

Culture	Control	Amount of filtrate added		
		1 drop	2 drops	2 cc
B. coli	−	−	−	−
Do	−	−	−	−
B. carotovorus	−	−	−	−
Do	−	−	−	−
B. melonis	−	−	−	−
Do	−	−	−	−
Cabbage-rot organism	−	±	±	±
Do	−	±	±	±

[a] The following symbols are used in the tables: −, very strong turbidity; ±, slightly less turbidity; +, cloudy; ++, less cloudy; +++, nearly clear; ++++, clear.

out. The collected liquid was filtered through a filter paper, previously impregnated with diatomaceous earth. The clear filtrate was passed through a Berkefeld filter to remove all the bacteria present. The technique used was similar to that recommended by d'Hérelle.[3] The organism was isolated and in addition stock strains of *Bacillus carotovorus*, *B. melonis*, and *B. coli* were now inoculated into tubes of plain broth and incubated for one hour. Varying amounts of the

[1] Received for publication Mar. 10, 1924.

[2] This work was done in the spring of 1922. Since writing this paper, the following article has appeared: GERRETSEN, F. C., AND OTHERS. DAS VORKOMMEN EINES BAKTERIOPHAGEN IN DEN WURZELKNÖLLCHEN DER LEGUMINOSEN. Centbl. Bakt., (II) 60: 311–316, illus. 1923.

[3] D'HÉRELLE, F., THE BACTERIOPHAGE, ITS RÔLE IN IMMUNITY. . . . Tr. by G. H. Smith. 287 p., illus. Baltimore. 1922.

sterile filtrate were then added to these tubes, as shown in Table I. The results were obtained in 48 hours. The tubes of the cabbage-rot organisms were now filtered as before and the filtrate again added to fresh broth cultures, with the results shown in Table II.

As Tables I, II, and III indicate, the second transfer shows a marked improvement in the inhibition of the organism and the third transfer shows an even more decided improvement over the second. In other words, a marked development of the inhibitory substance resulted from invigoration by transplanting.

TABLE II.—*The effect of the cabbage-rot organism after one invigoration*

Culture	Control	Amount of filtrate added		
		1 drop	2 drops	2 cc.
B. carotovorus	−	−	−	−
Do	−	−	−	−
Cabbage-rot organism	−	+	+	+
Do	−	+	+	+

[a] The following symbols are used in the tables: −, very strong turbidity; ±, slightly less turbidity; +, cloudy; ++, less cloudy; +++, nearly clear; ++++, clear.

In the same manner a third set was prepared with the following results (Table III):

TABLE III.—*The effect of the cabbage-rot filtrate after two invigorations* [a]

Culture	Control	Amount of filtrate added		
		1 drop	2 drops	2 cc.
Cabbage-rot organism	−	+	++	++++
Do	−	++++	++	++++

[a] The following symbols are used in the tables: −, very strong turbidity; ±, slightly less turbidity; +, cloudy; ++, less cloudy; +++, nearly clear; ++++, clear.

This substance is called an inhibitory substance because the tubes containing the filtrate remained clear for 48 hours and then slowly showed growth. The control tubes, on the other hand, were turbid in 24 hours. In no case was any evidence of lysis apparent, all the tubes showing only a retardation of growth for at least 48 hours.

The type of growth appearing in the tubes containing the filtrate was very interesting. The tubes after 48 hours would show a thin pellicle on the surface with a clear liquid beneath and no sediment. Upon agitation, this pellicle would break into granules, which would sink to the bottom of the tube. Upon shaking the tubes, the granules would break up and a persistent turbidity would result. The control tubes, on the other hand, would show a persistent turbidity in 24 hours without pellicle or sediment. The filtrate apparently induced this change of growth to take place.

Unpublished work by one of the authors (Mallmann) showed exactly the same occurrence in broth with *Bacillus coli* where an inhibitory substance of like nature was added. Plates from tubes of cabbage-rot organism showed only the typical normal colonies. These differ from the plates of *B. coli* where two types were found, a normal colony and a so-called "rough" colony, as shown by Bergstrand.[4]

After repeated transfers, the inhibitory substance adapted itself to some extent to other related organisms. It inhibited *Bacillus spieckermani* and *B. carotovorus*

[4] Bergstrand, H. ON THE VARIATIONS OF BACTERIUM COLI. *Jour. Bact. 8: 173-192, illus. 1923.*

TABLE IV.—*The effect of the cabbage-rot filtrate after repeated invigorations*[a]

Culture	Control	Amount of filtrate added		
		1 drop	2 drops	2 cc.
Cabbage-rot organism	−	++++	++++	++++
Do	−	++++	++++	++++
Bacillus spieckermani	−	−	−	++++
Do	−	−	−	++++
Bacillus carotovorus	−	−	−	++++
Do	−	−	−	++++
Potato-rot bacillus	−	−	−	−
Do	−	−	−	−

[a] The following symbols are used in the tables: −, very strong turbidity; ±, slightly less turbidity; +, cloudy; ++, less cloudy; +++, nearly clear; ++++, clear.

TABLE V.—*The effect of heating the filtrate at 56° C. for 30 minutes*[a]

Culture	Control	Amount of filtrate added		
		1 drop	2 drops	2 cc.
Cabbage-rot organism	−	+	++	++++
Do	−	±	±	±

[a] The following symbols are used in the tables: −, very strong turbidity; ±, slightly less turbidity; +, cloudy; ++, less cloudy; +++, nearly clear; ++++, clear.

TABLE VI.—*The effect of heating the filtrate at 63° C. for 30 minutes*[a]

Culture	Control	Amount of filtrate added		
		1 drop	2 drops	2 cc.
Cabbage-rot organism, unheated filtrate	−	++++	++++	++++
Do	−	++++	++++	++++
Cabbage-rot, heated filtrate	−	−	−	−
Do	−	−	−	−

[a] The following symbols are used in the tables: −, very strong turbidity; ±, slightly less turbidity; +, cloudy; ++, less cloudy; +++, nearly clear; ++++, clear.

where a large amount of filtrate was added, but refused to inhibit the potato-rot bacillus. A filtrate made two weeks later from these tubes failed to inhibit *B. spieckermani* and *B. carotovorus* but was still very active against the cabbage-rot bacillus.

RESISTANCE OF THE INHIBITORY SUBSTANCE TO HEAT

An active filtrate was heated to 56° C. for 20 minutes and then added as usual to the cabbage-rot bacillus.

The heating at 56° C. for 20 minutes decreases the activity of the inhibitory substance but it was still active enough to show partial inhibition in one case.

A second active filtrate was now heated to 63° C. for 30 minutes and added as usual to young organisms in broth.

This temperature caused a complete destruction of the inhibitory substance as shown in the above table. This checks very favorably with previous work by others working on similar substances isolated from animal sources.

CONCENTRATION OF THE INHIBITORY SUBSTANCE

An active filtrate was added to broth cultures of young cabbage-rot bacillus in dilutions starting with 1 to 10 and progressing by dilutions of 10 up through 12 dilutions, or to a final dilution of 1 to 1,000,000,000,000. The tubes of broth contained 9 cc. of broth. To the first tube was added 1 cc. of filtrate making a 1-to-10 dilution. Using the same pipette, 1 cc. was taken from this tube to the second, and so on through the set. All of these tubes showed inhibition through a dilution of 1 to 100,000,000,000.

After a period of several months, the above experiment was repeated, using higher dilutions. The dilutions this time were run up by dilutions of 10 to a final dilution of 1 to 1,000,000,000,000,000,000. The filtrate still showed inhibition at a dilution of 1 to 100,000,000,000,000,000. The last dilution failed to show any inhibition.

The dilution in which the inhibitory substance is active is extremely high, far higher than could be induced through any toxic material produced by the organism. There is but little doubt that the material isolated is comparable to inhibitory substances obtained from animal sources.

The organism upon which the inhibitory substance was active proved upon identification to belong to the fluorescent group rather than *Bacillus carotovorus*.[5] However, this organism does decompose cabbage. Experiments to determine its decomposing power showed a slow rotting, several weeks being required for complete liquefaction of the cabbage.

Filtrates made from the pure culture never showed the inhibitory substance nor did the culture of *Bacillus carotovorus*. This proves that the inhibitory substance must have come from the cabbage. Filtrates from normal cabbage failed to show the inhibitory substance, which indicates an association of the inhibiting substance with the disease-producing organism. This is similar to conditions in the intestinal tract of animals as demonstrated by d'Hérelle.[6]

CONCLUSIONS

An inhibitory substance was isolated from a rotten cabbage which was active on an organism obtained from the same cabbage.

The inhibitory substance became active against other soft rot-producing organisms but this activity was lost by further transplanting.

The inhibitory substance was not destroyed at 56° C. for 20 minutes, but was destroyed at 63° for 30 minutes, showing a sensitiveness to heat comparable to microorganisms and lytic substances isolated from animal sources.

The inhibitory substance was present in extremely large amounts, as indicated by its activity in high dilution. It was therefore probably not a toxic product of the organism.

Lytic and inhibitory substances are probably found in plants as in animals. It is hoped that this brief preliminary report will open up this field and show the extent of lytic and inhibitory substances in the plant world.

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[5] The organism is approximately the same size and shape as *Bacillus carotovorus*. It occurs singly and is sluggishly motile; Gram-negative, no gas produced in dextrose, lactose, or saccharose broth. Plain broth becomes decidedly turbid in 24 hours with a slight pellicle appearing in six to seven days, at which time a yellowish green coloration appears near the surface. The agar slant shows an abundant white opaque growth with no discoloration of the medium. Gelatin is liquefied.

[6] D'HÉRELLE, F., THE BACTERIOPHAGE, ITS RÔLE IN IMMUNITY. . . . tr. by G. H. Smith. 287 p., illus Baltimore. 1922.